DECLARATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Toshio SUZUKI et al.

Serial No. 10/022,619: Group Art Unit: 1614

Filed: December 20, 2001: Examiner: Sandra E. Saucier

For: PROCESS FOR PREPARATION OF (R)-1,2-PROPANEDIOL BY MICROBES

Honorable Commissioner of Patents and Trademarks

Sir:

I, Toshio SUZUKI, hereby declare and state as follows:

1. I am one of inventors of the present invention.

2. I received my undergraduate education at the Biology Course of the Faculty of Science, Osaka City University in 1986, and then my MS from the graduate school of the Faculty of Agricultural Chemistry, Osaka Prefecture University in 1988. I received my Ph. D. from Osaka City University for thesis "Study on microbial and enzymatic dehalogenation of haloalcohol" in 1994.

3. I have been employed by Daiso Co., Ltd. Since 1988, I have been engaged in this corporation, assigned in Research laboratory, and have had a total 15-year research and development experience in microbial and enzymatic conversion of organic compounds, especially optically

active halogenated compounds.

4. I am a member of both The American Chemical Society and The Society for Bioscience and Bioengineering, Japan.

5. I was awarded the Industrial Technology Prize in 1996 from The Society for Bioscience and Bioengineering, Japan and the Invention Prize in Kinki area from the Japan Institute of Invention and Innovation in 1996.

The following experiments were carried out by me.

Estimation of strains between Cited references, i.e. JP 6-209781 and JP 6-030790 and the present invention.

1. Purpose

Between strains of Cited references and the present invention, I estimated ability of the asymmetric assimilation of the respective bacterial strains, belonging to Pseudomonas, using the complete synthetic medium containing (RS)-1,2-propanediol (abbreviated as PG) as a single carbon source. Also, I estimated their ability of asymmetric assimilation using the complete synthetic medium containing (RS)-3-chloro-1,2-propanediol (abbreviated as CPD) as a single carbon source, instead of (RS)-PG.

2. Methods

1) Strains tested: four strains of Cited references; Pseudomonas putida TRB-2, Pseudomonas putida TRP-4, Pseudomonas putida RP-7 and Pseudomonas sp. TRP-13, and

a strain of the present invention; Pseudomonas nitroreducens DS-S-RP8.

2) Medium: The liquid complete synthetic medium comprised several amounts (%(v/v)) of (RS)-1,2-propanediol as a single carbon source, 1 %(w/v) of $(NH_4)_2SO_4$ as an inorganic nitrogen source, 0.02 %(w/v) of Na_2HPO_4 $12H_2O$, 0.04 %(w/v) of NaH_2PO_4 $2H_2O$, 0.02 %(w/v) K_2HPO_4, trace of metals (0.001-0.0001% (w/v)) (Mn^{2+}, Cu^{2+} and Fe^{2+}) and 1% (w/v) of $CaCO_3$ as a neutralized agent (pH 6.8). In case of asymmetric assimilation for (RS)-CPD, (RS)-CPD was added as a single carbon source instead of (RS)-PG. And then, 100 ml each of this medium was discarded into a 500-ml Erlenmeyer flask, sterilized by autoclaving at 121°C for 15 min. and used as the complete synthetic medium for the asymmetric assimilation test.

3) Cultivation: Cultivation of respective strains was carried out as follows; first, the respective strains were cultured on an agar plate of a common nutrient medium containing 0.5 % (w/v) each of peptone and yeast extract (pH7.0) for 24hr at 30°C. Second, a small loopful of the cultured cells was suspended into 5 ml of the above complete synthetic medium and 0.5 to 2 ml amount of the cell suspension as a starter cell was inoculated into the above 100 ml of the complete synthetic medium, cultured aerobically at 30°C with vigorous shaking. This time, I

planned to carried out the asymmetric assimilation tests at the concentration of 2 %(v/v) and 2 to 4 %(v/v) of (RS)-PG. Third, after 24 to 48 (72) hr cultivation, degradation of (RS)-PG or (RS)-CPD and cell growth were determined. The degradation was analyzed by gas chromatography and cell growth was turbidimetrically estimated at 660 nm using spectrophotometer instrument. If the degradation proceeds, optical purity was determined and production ability of optically active PG at several concentration of (RS)-PG was estimated by the asymmetric assimilation method.

4) Result

Table 1: Estimation of ability of the asymmetric assimilation at 2%(v/v) (RS)-PG as a single carbon source

Strain	Cell growth (OD at 660 nm) (24hr/48hr/72 hr)	Degradation (%)* (24hr/48hr/72hr)	Optical purity (%ee) (72 hr)
TRB-2	0.80/3.76/4.42	2.89/53.9/77.9	>99.9(R)
TRP-4	0.60/0.46/0.60	2.85/11.5/35.4	ND
TRP-7	0.30/0.16/0.38	4.40/18.5/23.2	ND
TRP-13	0.28/0.34/0.32	0.35/10.0/10.3	ND
DS-S-RP8	0.54/2.66/3.66	8.07/47.8/78.2	>99.9(R)

*) Degradation was estimated to be taken 0% at initial time.

As a starter, a 0.5ml amount of the cell suspension was inoculated into 100 ml of the complete synthetic medium.

Table 2-1: Cell growth at several % (RS)-PG as a single carbon source (cell growth)

Cell growth (OD at 660 nm) (24hr/48hr)					
Conc. (%(v/v))	TRB-2	TRP-4	TRP-7	TRP-13	DS-S-RP8
2	3.60/5.60	0.02/0.06	0/0.02	0.16/0.50	2.74/4.20
4	1.80/2.00	0/0.02	0/0.02	0.14/0.46	4.44/9.02

As a starter, a 2ml amount of the cell suspension was inoculated into 100 ml of the complete synthetic medium.

Table 2-2: Estimation of ability of the asymmetric assimilation at several % (RS)-PG as a single carbon source (degradation and optical purity)

Degradation(%)* (24hr/48hr); Optical purity (%ee at 48 hr)					
Conc. (%(v/v))	TRB-2	TRP-4	TRP-7	TRP-13	DS-S-RP8
2	23.5/77.7 (99.1%ee)	3.19/21.1 ND	0/2.75 ND	8.98/14.3 ND	18.9/57.9 (97.5%ee)
4	13.8/42.4 (60.3%ee)	4.19/16.6 ND	9.41/8.76 ND	0/6.95 ND	27.4/66.7 (99.3%ee)

*) Degradation was estimated to be taken 0% at initial time.

As a starter, a 2ml amount of the cell suspension was inoculated into 100 ml of the complete synthetic medium.

Table 3: Estimation of ability of the asymmetric assimilation at 2%(v/v) (RS)-CPD as a single carbon source

Strain	Strain Growth OD at 660nm, (24hr/48hr48hr/72hr)	Degradation*(%) (24hr/48hr/72hr)
TRB-2	0.16/0.16/-	0/7.33/-
TRP-4	0.20/0.12/-	0/0.36/-
TRP-7	0.16/0.12/-	0/0.60/-
TRP-13	0.18/0.12/-	0/0.77/-
DS-S-RP8	1.62/2.34/3.42	9.63/20.1/48.5**

*) Degradation was estimated to be taken 0% at initial time.

**) Optical purity: 96.1% e.e.(S-form)

As a starter, a 2ml amount of cell suspension was inoculated into 100 ml of the complete synthetic medium.

5) Discussion and conclusion

Only one strain TRB-2 of four strains of Cited references has ability of the asymmetric assimilation of (RS)-1,2-propanediol (PG), but the rest of three strains have no symmetric assimilation ability for PG (Table 1).

Although strain TRB-2 of Cited references has asymmetric assimilation ability at lower concentration of (RS)-PG, the ability was not available at higher concentration (4 %(v/v)) (Table 2).

On the other hand, strain DS-S-RP8 of the present invention has higher ability than that of strain TRB-2. At 4 %(w/v) PG concentration, strain DS-S-RP8 grew faster and more than strain TRB-2, so that degradation of PG of strain DS-S-RP8 was higher than strain TRB-2. At that time, optical purity was estimated to be >99 %ee (strain DS-S-RP8 of the present invention) and 60.2 %ee (strain TRB-2 of Cited references) (Tables 2-1, 2-2).

Moreover, all strains of Cited references have no asymmetric assimilation ability of (RS)-3-chloro-1,2-propanediol (CPD) with releasing chloride ion (Table 3).

On the other hand, a strain, DS-S-RP8 of the present invention has the asymmetric assimilation ability of both PG and CPD (Table 1 and Table 3), and in particular, showed excellent potential for production of (R)-PG by the asymmetric assimilation method at even higher concentration (4 %(v/v)) (Table 2).

Strains between Cited references and the present invention are different in their strain level, except that one strain, TRB-2 has asymmetric assimilation ability of PG. However strain TRB-2 has no asymmetric assimilation ability of CPD and is far inferior to strain DS-S-RP8 of the present invention in assymetric assimilation ability at higher concentration (e.g. 4%) of PG.

Therefore, Pseudomonas sp. DS-S-RP8 of the present invention is quite different from strains (all of which belong to genus Pseudomonas) disclosed in Cited references in their strain level.

The undersigned declares further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1000 of Title 18 of the United State Code and that

such willful false statement may jeopardize the validity of the above mentioned application or any patenting thereon.

This 14th day of November, 2003

Toshio SUZUKI